JOURNAL OF BACTERIOLOGY, Aug. 1991, p. 4773–4781
0021-9193/91/154773-09$02.00/0

Vol. 173, No. 15

Purification and Characterization of Thin, Aggregative Fimbriae from *Salmonella enteritidis*

S. KAREN COLLINSON,[1] LEVENTE EMÖDY,[2] KARL-HEINZ MÜLLER,[1] TREVOR J. TRUST,[1] AND WILLIAM W. KAY[1]*

Department of Biochemistry and Microbiology, Petch Building, P.O. Box 3055, University of Victoria, Victoria, British Columbia, V8W 3P6, Canada,[1] and Institute of Microbiology, University Medical School, Pécs, H-7643, Hungary[2]

Received 19 December 1990/Accepted 20 May 1991

Novel fimbriae were isolated and purified from the human enteropathogen *Salmonella enteritidis* 27655. These fimbriae were thin (measuring 3 to 4 nm in diameter), were extremely aggregative, and remained cell associated despite attempts to separate them from blended cells by centrifugation. The thin fimbriae were not solubilized in 5 M NaOH or in boiling 0.5% deoxycholate, 8 M urea, or 1 to 2% sodium dodecyl sulfate (SDS) with or without 5% β-mercaptoethanol. Therefore, an unconventional purification procedure based on the removal of contaminating cell macromolecules in sonicated cell extracts by enzymatic digestion and preparative SDS-polyacrylamide gel electrophoresis (PAGE) was used. The insoluble fimbriae recovered from the well of the gel required depolymerization in formic acid prior to analysis by SDS-PAGE. Acid depolymerization revealed that the fimbriae were composed of fimbrin subunits, each with an apparent molecular mass of 17 kDa. Although their biochemical characteristics and amino acid composition were typical of fimbriae in general, these thin fimbriae were clearly distinct from other previously characterized fimbriae. Moreover, their fimbrin subunits had a unique N-terminal amino acid sequence. Native fimbriae on whole cells were specifically labeled with immune serum raised to the purified fimbriae. This immune serum also reacted with the denatured 17-kDa fimbrin protein in Western blots. The polyclonal immune serum did not cross-react with the other two native fimbrial types produced by this strain or with their respective fimbrins on Western blots (immunoblots). Therefore, these fimbriae represent the third fimbrial type produced by the enteropathogen *S. enteritidis*.

Fimbriae (also called pili) comprise a class of nonflagellar, proteinaceous filaments produced on the surface of a wide range of bacteria (11, 43, 46). Detailed studies of several fimbriae from a variety of animal and human pathogens have revealed these appendages to be highly organized, complex structures which in some cases facilitate bacterial adherence to specific host tissues (21, 45, 46, 56). Fimbriae are polymers composed mainly of a single protein species named fimbrin, which usually forms filaments 2 to 7 nm wide. Two general groups of fimbriae are differentiated by the presence or absence of *N*-methylphenylalanine (N-MePhe) as the N-terminal amino acid residue on their respective fimbrins (46). Important nonenteric pathogens including *Pseudomonas* spp., *Neisseria* spp., and *Moraxella* spp. produce N-MePhe-containing fimbriae, whereas the fimbriae which are widespread among members of the family *Enterobacteriaceae* do not contain this residue. The best-characterized adhesive fimbriae among the *Enterobacteriaceae* have been isolated from *Escherichia coli*. Type 1 fimbriae, produced by pathogenic and nonpathogenic *E. coli*, are rigid and 6 to 7 nm wide. They have an additional minor fimbria-associated adhesin protein which confers on them the mannose-sensitive adherence characteristic (11, 46). Since these fimbriae mediate the agglutination of erythrocytes except in the presence of D-mannose, likely this carbohydrate forms the type 1 fimbrial receptor on eukaryotic cells (11). Pap fimbriae, formed by uropathogenic *E. coli*, are morphologically similar to type 1 fimbriae but exhibit mannose-resistant hemagglutination, since the adhesins recognize the α-D-galactose-(1-4)-β-D-galactose moiety of glycolipid receptors found on human uroepithelial cells (56). Similarly, *E. coli* responsible for neonatal meningitis produce a second class of mannose-resistant fimbriae, S fimbriae, whose adhesins recognize sialylgalactoside-containing receptors (56). Another important group of mannose-resistant fimbriae include those characterized from various host-adapted enterotoxigenic *E. coli* (ETEC) which enhance bacterial colonization of the epithelial mucosa of the small intestine, thereby enabling these bacteria to establish infections by avoiding host clearance mechanisms (21, 27). These plasmid-encoded fimbriae are either rigid filaments 6 to 7 nm wide or thin, flexible fibrils 2 to 3 nm in diameter (21, 34). In addition, some *E. coli* produce thin (2-nm), coiled fibrils named curli, which are noted for their ability to mediate bacterial adherence to fibronectin (42). Numerous other fimbriae in various *E. coli* strains have been described but have yet to be biochemically characterized in detail (8, 10, 14, 24, 55, 58).

Salmonella spp., a related genus of important enteropathogens, also produce mannose-sensitive type 1 fimbriae which are morphologically similar but antigenically distinct from those of *E. coli* (11, 29, 48). However, the role of these fimbriae in enteric disease is not clear (20, 53). Some *Salmonella* spp. produce nonhemagglutinating type 2 fimbriae which exhibit type 1 morphology (11). Recent evidence indicates these are actually type 1 fimbriae lacking their associated adhesin (13). Type 3 fimbriae, observed on some *Salmonella* spp. as well as on other *Enterobacteriaceae*, are thin filaments 2 to 4 nm in diameter and are notable for their ability to agglutinate tannic acid-treated erythrocytes (1, 2, 40). Like other fimbriae in *Salmonella* spp. (62), type 3 fimbriae have not been biochemically characterized in detail.

Salmonella enteritidis is an important foodborne enteric pathogen whose worldwide incidence is increasing signifi-

* Corresponding author.

cantly (12, 50, 57). This increase has been due in part to the ability of *S. enteritidis* to infect chicken oviducts, thereby contaminating the contents of intact eggs (26). In view of the potential importance of fimbriae in *Salmonella* pathogenesis, we have been characterizing various fimbrial types from *S. enteritidis* 27655, an enterotoxigenic strain originally isolated from a patient in India (6). This strain produces three biochemically and serologically distinct fimbriae. The previously characterized fimbriae include those composed of 14-kDa fimbrin subunits (17, 18), which were recently renamed SEF 14 (*S. enteritidis* fimbriae composed of 14-kDa fimbrin), as well as the mannose-sensitive type 1 fimbriae composed of 21-kDa fimbrin subunits (SEF 21) (37a). In this study we report that *S. enteritidis* 27655 also produces unusual thin, aggregative fimbriae which were found to be extremely insoluble and recalcitrant to purification and characterization by biochemical methods conventionally used for fimbriae.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. enteritidis* 27655 strain 3b used for this study was the same strain as *S. enteritidis* 27655-3b and *S. enteritidis* 3b referred to in previous publications (17–19, 38). *S. enteritidis* was routinely grown at 37°C for 20 to 24 h on T medium containing 1% tryptone (Difco Laboratories, Detroit, Mich.) adjusted to pH 7.2 and solidified with 1.5% agar. Cultures were grown at 30°C for 48 h in static liquid colonization factor antigen (CFA) medium (16) containing 12 mM Na_2HPO_4 and 5 mM KH_2PO_4.

Isolation and purification of aggregative fimbriae. *S. enteritidis* was scraped from 10 T-medium plates and suspended in 30 ml of 10 mM Tris HCl, pH 8.0 (Tris buffer), supplemented with 0.1 mg of RNase A (bovine pancreas; Sigma Chemical Co., St. Louis, Mo.) and 0.1 mg of DNase I (bovine pancreas; Boehringer, Mannheim, Germany) per ml. The cells were broken by sonication, and $MgCl_2$ was added to the resulting sonic extract to 1 mM prior to incubation at 37°C for 20 min. Lysozyme (Sigma) was added to 1 mg/ml, and the samples were incubated with shaking (40 min, 37°C), after which they were adjusted to 1% sodium dodecyl sulfate (SDS) and incubated further (30 min, 37°C). The remaining insoluble material was collected by centrifugation (12,100 × *g*, 15 min, 25°C), washed and suspended in 10 ml of Tris buffer, boiled to melt contaminating agar present in the sample, centrifuged, and boiled to remove any residual agar. The pellet was digested again with RNase, DNase, and lysozyme as described above; washed twice with Tris buffer; and suspended in 2 ml of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (10% glycerol, 5% β-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl [pH 6.8]) (32). The sample was boiled for 15 min, loaded onto a preparative 12% polyacrylamide gel (3% stacking gel), and subjected to electrophoresis at 20 mA for 5 h. The material which did not enter the stacking gel was recovered, washed three times in distilled deionized H_2O, extracted twice with 95% ethanol, and lyophilized. The dried material was resuspended in distilled deionized H_2O and sonicated to break up the large clumps. The insoluble material was extracted with 0.2 M glycine (pH 1.5; adjusted with HCl) at 100°C for 10 min, recovered by centrifugation (16,000 × *g*, 10 min, 4°C), washed five times with distilled deionized H_2O, lyophilized, and stored at −20°C.

Electrophoresis. SDS-PAGE was performed according to the method of Laemmli (32) as modified by Ames (3). Protein samples were usually boiled for 10 min in SDS-PAGE sample buffer prior to electrophoresis on 12% gels poured with a 5% stacking gel. However, the aggregative fimbriae required an additional pretreatment so that the protein would enter the gel. The insoluble fimbriae were mixed for a few seconds with 90% formic acid, immediately frozen, and then lyophilized to remove the acid. Alternatively, the formic acid was removed by using a Speed Vac concentrator (Savant Instruments Inc., Hicksville, N.Y.) operated at 45°C for 1 h. The acid-treated sample was then resuspended in SDS-PAGE sample buffer and subjected to electrophoresis. PAGE for the separation of peptides was performed by the method of Swank and Munkres (54), and the polyacrylamide isoelectric focusing gels were dealt with as described by Robertson et al. (49).

Preparation of immune serum. Purified, insoluble, aggregative fimbriae (400 μg) were suspended in phosphate-buffered saline (pH 7.2) (51), sonicated, and emulsified with Freund's complete adjuvant prior to subcutaneous and intramuscular injections of a 1-month-old female New Zealand White rabbit. The rabbit was subsequently boosted at 3 and 5 weeks with an additional 400 μg of protein emulsified with Freund's incomplete adjuvant. Titers of the immune serum were determined by enzyme-linked immunosorbent assay (15) with sonicated aggregative fimbriae as the antigen and goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Caltag Laboratories, San Francisco, Calif.) as the second antibody. The assay was developed with 1 mg of *p*-nitrophenyl phosphate per ml in diethanolamine buffer (0.97 M diethanolamine, 0.5 mM $MgCl_2$, 0.02% NaN_3; pH 9.5). Preimmune serum was collected 1 week prior to the first immunization.

Western blot (immunoblot) analysis. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose by using an LKB Multiphor II Electrophoresis System (LKB-Pharmacia, Broma, Sweden) and the recommended discontinuous buffer system. The nitrocellulose membranes were incubated in 20 mM Tris (pH 7.5)–0.25 M NaCl–0.05% Tween 20 (TBS-Tween 20) and 3% skim milk and then incubated with immune serum to the aggregative fimbriae (diluted 1/1,000 in TBS-Tween 20). Finally, the membranes were incubated with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (diluted 1/1,000 in TBS-Tween 20 containing 1.5% skim milk). The Western blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as previously described (38).

Electron microscopy. *S. enteritidis* cells or purified fimbriae deposited on Formvar-coated copper grids were negatively stained with 1% ammonium molybdate containing 0.1% glycerol. For immunogold labeling of *S. enteritidis* fimbriae, grids coated with bacteria or fimbriae were incubated on a drop of 10 mM Tris (pH 8.0)–0.15 M NaCl–1% skim milk, transferred to a drop of preimmune or immune serum (diluted 1/1,000 in Tris-NaCl buffer containing 0.1% skim milk), washed in Tris-NaCl buffer, and floated on a drop of protein A–15-nm gold (Auroprobe; Pharmacia, Uppsala, Sweden) (diluted 1/50 in Tris-NaCl buffer containing 0.1% skim milk). The grids were rinsed, negatively stained as described above, air dried, and observed with a Philips EM300 electron microscope operated at 60 or 80 kV.

Amino acid analyses. A sample of the purified, aggregative fimbriae was suspended in 90% formic acid, and the N-terminal sequences on the solubilized fimbriae were determined by using an Applied Biosystems model 470A gas-phase sequencer with on-line phenylthiohydantoin analysis. The N-terminal amino acid sequence was confirmed by using the

major 17-kDa protein band separated by SDS-PAGE and electrophoretically transferred to Immobilon (Millipore Corp., Bedford, Mass.). The Immobilon-bound 17-kDa protein was stained with Coomassie blue R-250 (Sigma), cut from the membrane, and sequenced directly (33). Comparisons of the N-terminal sequences of the aggregative fimbriae with proteins listed in the GenBank data base (release number 64), SWISS-PROT (release number 14), and GENPEPT (release number 63) were made by using the FASTA program (47).

Total amino acid analysis of the purified fimbriae was obtained on samples hydrolyzed in gaseous HCl (165°C, 1 h) and analyzed by using an Applied Biosystems model 420 amino acid derivatizer analyzer. The cysteine content was determined on carboxymethylated samples of the formic acid-treated fimbriae (28). Derivatized proteins were removed from the reaction mixture by overnight incubation (4°C, dark) in the presence of small pieces of Immobilon. The nylon membrane-bound proteins were removed from the mixture, rinsed, air dried, and stored at −20°C until analysis. β-Lactoglobulin was used as a standard to confirm the success of the carboxymethylation reaction.

RESULTS

Identification and purification of aggregative fimbriae. When *S. enteritidis* was grown for 24 h on T medium, solid colonies were produced rather than the normal mucoid colonies seen when this strain was grown on other solid media. Electron microscopic observation of negatively stained *S. enteritidis* isolated from T medium revealed that cells were usually, but not exclusively, arranged in clumps which often had copious amounts of a fibrillar structure closely associated with the cells (Fig. 1A). The individual fimbria were thin (3 to 4 nm in diameter) and flexible and tended to aggregate (Fig. 1B). Initial attempts to separate this fibrillar material from blended cells by low-speed centrifugation resulted in the presence of flagella and some fimbriae with type 1 morphology in the supernatant, whereas the aggregative fimbriae remained clumped and chiefly cell associated (Fig. 1C). Similarly, the treatment of the cells at 70°C for 30 min or the disruption of cells with either a French pressure cell or ultrasound did not facilitate the separation of this fibrillar material from cells, since it consistently pelleted with the cell debris during subsequent centrifugation steps.

Our attempts to solubilize the structure from the cell surface by using various chemical treatments indicated that the structure was very resistant to disaggregation, denaturation, and depolymerization. Electron microscopic observation revealed that the fimbriae were not obviously disrupted by incubation for several hours in 5 M NaOH or by boiling in 1% SDS, 0.5% deoxycholate, or SDS-PAGE sample buffer containing 2% SDS and 5% β-mercaptoethanol. The aggregative fimbriae could be recovered, apparently intact, from the well of a polyacrylamide gel following electrophoresis of whole-cell lysates (data not shown). Consequently, the purification strategy for these fimbriae involved removal of all other cell macromolecules by differential solubilization, enzymatic digestion, and electrophoresis. Electron microscopic observation of the fimbriae purified in this manner indicated that they were free of obvious cellular debris and were morphologically identical to the fibrils seen initially on the cell surface but were arranged in compact aggregates (Fig. 1D). Approximately 12 to 14 mg of the aggregative fimbriae were recovered from cells harvested from 10 petri plates (9-cm diameter). This corresponded to approximately 20 mg of fimbriae per 10^{12} cells.

Electrophoresis was initially used to monitor the presence of the fimbriae during the various purification stages. Virtually all of the contaminating cellular protein and added enzymes were separated from the fimbriae during SDS-PAGE, since the latter remained in the well of the preparative gel as a white precipitate (Fig. 2A, compare lanes 4 and 5). When 24 μg of the insoluble white precipitate was applied to a gel, virtually no protein was detected in the stacking or separating gel by Coomassie staining (Fig. 2A, lanes 5 and 7) or silver staining. Moreover, samples heated to 100°C for 10 min in SDS-PAGE sample buffer supplemented with 8 M urea or acidified with 0.2 M glycine (pH 1.5) did not result in detectable protein bands on urea-containing gels or SDS-polyacrylamide gels, respectively (data not shown). However, brief pretreatment of the insoluble fimbriae with 90% formic acid prior to electrophoresis resulted in the resolution of a single, major protein band of approximately 17 kDa separated from two minor protein bands of 15 and 33 kDa (Fig. 2A, lane 8). Similarly, Western blot analysis revealed that the major immunoreactive protein failed to migrate into the stacking gel in spite of the various treatments incurred during the course of purification unless the fimbriae were pretreated with formic acid (Fig. 2B, compare lanes 1 and 2). The preimmune serum contained immunoglobulins weakly cross-reactive with a few *S. enteritidis* proteins and lipopolysaccharide which were visualized in overdeveloped Western blots (data not shown). However, these protein and lipopolysaccharide contaminants were also removed from the fimbriae during the preparative electrophoresis step and were not evident in Western blots of subsequent purified samples. In addition to the detection of the major 17-kDa protein and the minor protein bands present in the purified sample, the immune serum raised to the purified fimbriae detected some high-molecular-mass immunoreactive bands on Western blots of the formic acid-treated fimbriae (Fig. 2B, lane 2). These additional protein bands likely represented multimers of the 17-kDa fimbrin, since acid-treated samples boiled in sample buffer prior to electrophoresis revealed only the 17-kDa band and a faint 33-kDa band in Coomassie-stained gels and Western-blotted gels. This additional boiling treatment also appeared to render the fimbriae partially insoluble, since the 17-kDa protein band was less prominent on gels (Fig. 2A, compare lanes 8 and 9) and on Western blots (Fig. 2B, compare lanes 2 and 4). This problem was exacerbated when protein concentrations exceeded 2 μg/μl of SDS-PAGE sample buffer.

The possibility that the major fimbrial protein was being hydrolyzed by the formic acid pretreatment was tested by incubating purified fimbriae in concentrated formic acid for 3 h at 50°C prior to evaporation of the formic acid instead of subjecting the fimbriae to the usual brief exposure to the acid prior to immediate freezing and lyophilization. Both treatments resulted in similar ratios of the major 17-kDa protein and the minor 15- and 33-kDa proteins on gels. Notably, no obvious hydrolysis products were apparent when the formic acid-treated fimbriae were analyzed on polyacrylamide gels containing 2.5 or 8 M urea for the separation of low-molecular-mass peptides (data not shown). Moreover, N-terminal amino acid sequence analysis of the formic acid-treated fibrillar structure indicated the presence of a single protein species with traces of contaminating protein making up less than 1% of the sample.

These results indicated that the aggregative fimbriae of *S. enteritidis* were composed of polymerized fimbrin subunits

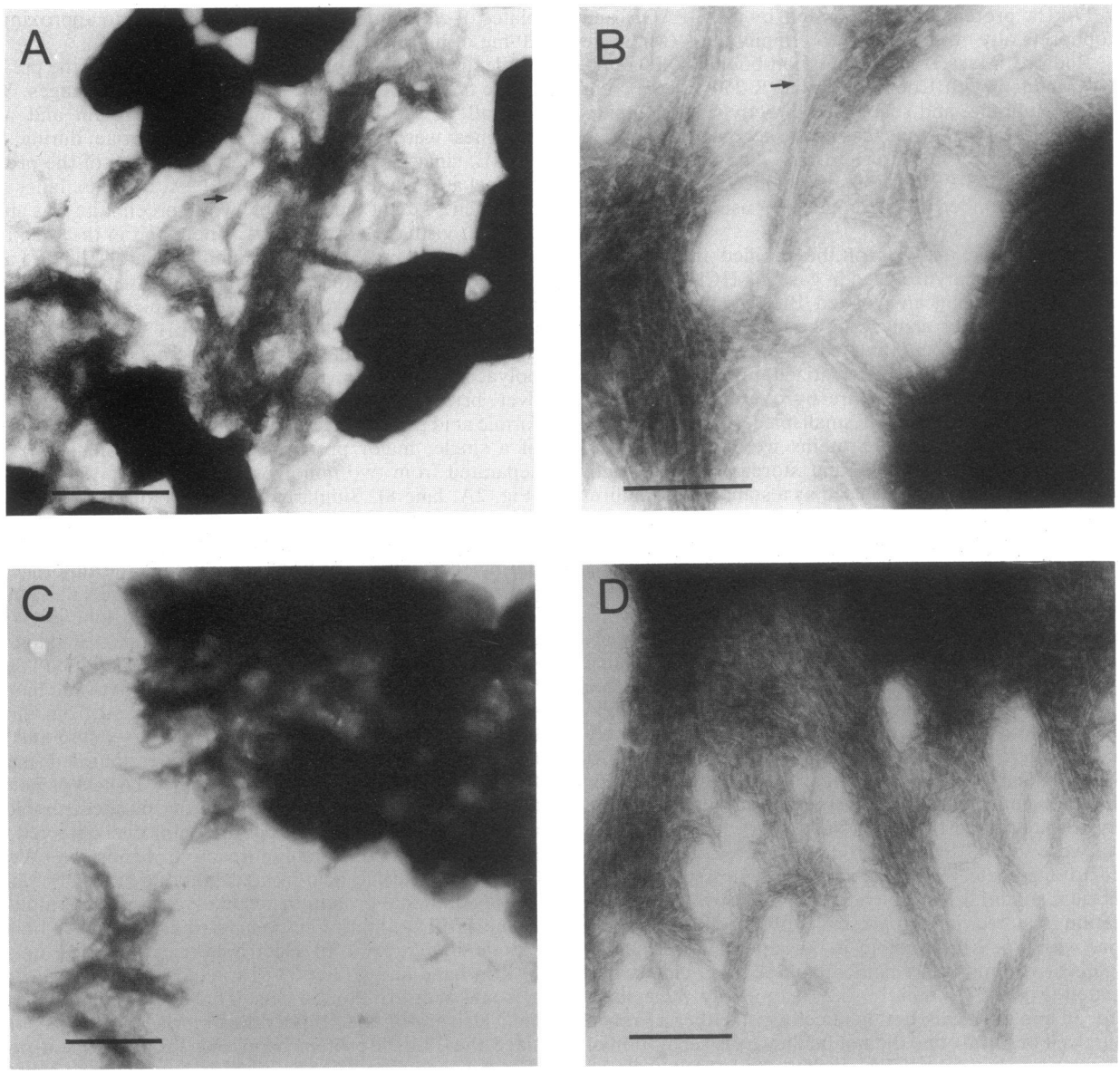

FIG. 1. Electron micrographs of negatively-stained *S. enteritidis* 27655-3b cells exhibiting thin, aggregative fimbriae. (A) Cells isolated from T medium. Arrow indicates a flagellum. Bar, 1.0 μm. (B) Enlargement of the cell-associated fimbriae. Arrow indicates a flagellum. Bar, 200 nm. (C) Blended cells recovered from the cell pellet after centrifugation. Bar, 1.0 μm. (D) Purified thin, aggregative fimbriae. Bar, 200 nm.

with apparent molecular masses of 17 kDa. Therefore, according to the nomenclature scheme developed for *S. enteritidis* fimbriae (37a), these fimbriae would be named SEF 17 (*S. enteritidis* fimbriae with a fimbrin molecular mass of 17 kDa).

Amino acid analysis and isoelectric focusing. The N-terminal amino acid sequence of the purified, formic acid-treated aggregative fimbriae and the 17-kDa protein band isolated following SDS-PAGE were identical (Table 1). The FASTA N-terminal sequence comparison between the 17-kDa fimbrin and protein sequences listed in the GenBank data bases did not reveal any other fimbrial proteins with sequences similar to those of the aggregative fimbriae of *S. enteritidis*.

The total amino acid analysis of the aggregative fimbriae gave the same results for samples taken before and after glycine extraction and before and after formic acid treatment. The fimbriae purified from *S. enteritidis* were composed of 34% hydrophobic residues and contained no detectable cysteine and very little methionine (Table 2). The total potential acidic residues constituted 30% of the total protein.

Isoelectric focusing of the purified, formic acid-treated fimbriae resulted in the presence of multiple bands, probably due to fimbrin isoforms. Native gels revealed three bands of equal intensity with acidic pI values (4.4, 4.6, and 4.8), whereas denaturing isoelectric focusing gels showed two bands, one of pI 4.6 and a single, less intensely staining band of pI 4.8 (data not shown).

Immunochemistry. Immunogold labeling of *S. enteritidis* cells revealed that the immune serum raised to the purified fimbriae recognized the aggregative fimbriae present on the

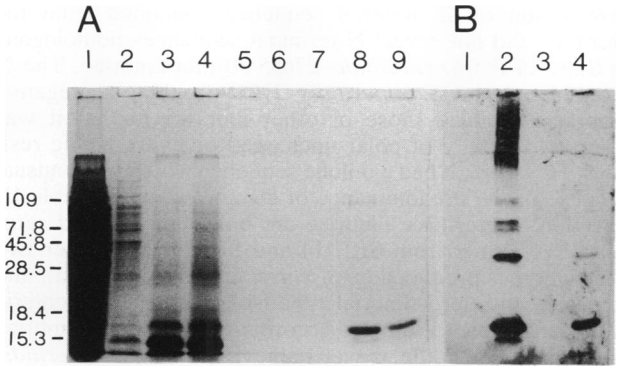

FIG. 2. (A) SDS-PAGE of various samples taken during the purification of the thin, aggregative fimbriae isolated from *S. enteritidis* 27655-3b. Lanes: 1, sonicated whole cells; 2, sample following first DNase-RNase-lysozyme-SDS treatment; 3, sample following second DNase-RNase-lysozyme treatment; 4, sample boiled in SDS-PAGE sample buffer prior to preparative electrophoresis; 5, insoluble material recovered from well of preparative gel after electrophoresis (24 μg); 6, 0.2 M glycine (pH 1.5) extract of the insoluble material; 7, purified fibrillar structure (24 μg); 8, formic acid-treated fibrillar structure not boiled prior to electrophoresis (12 μg); 9, formic acid-treated fibrillar structure boiled prior to electrophoresis (12 μg). Lanes 1 to 5 and 7 received 0.05% (by volume) of total preparation, whereas lane 6 received 0.5%. (B) Western blot analysis of purified aggregative fimbriae. Lanes: 1, not pretreated with formic acid and not boiled prior to electrophoresis; 2, pretreated with formic acid but not boiled prior to electrophoresis; 3, not pretreated with formic acid but boiled for 10 min prior to electrophoresis; 4, pretreated with formic acid and boiled for 10 min prior to electrophoresis. Each lane received 5 μg of protein. The molecular masses of prestained protein standards (Bethesda Research Laboratories, Bethesda, Md.) are indicated on the left as follows: phosphorylase *b*, 109 kDa; bovine serum albumin, 71.8 kDa; ovalbumin, 45.8 kDa; carbonic anhydrase, 28.5 kDa; β-lactoglobulin, 18.4 kDa; lysozyme, 15.3 kDa.

cell surface and found in regions between the cells (Fig. 3A, inset). This confirmed that the purified, aggregative fimbriae (SEF 17) had the same fibrillar structure originally identified on and isolated from these cells. Since *S. enteritidis* grown on T medium produces low amounts of its other two fimbrial types, *S. enteritidis* was grown in static CFA broth at 30°C to promote the expression of type 1 fimbriae (SEF 21) (37a) to see if these native fimbriae would cross-react with immune serum raised to SEF 17. Some cells grown under these conditions produced no fimbriae, some produced only SEF 21, and others appeared to produce both SEF 21 and the thin, aggregative fimbriae. However, only the last fimbriae were labeled with immune serum raised to SEF 17, as detected with immunogold (Fig. 3B). Similarly, a purified preparation of the previously characterized SEF 14 fimbriae (17, 18) was not labeled with the immune serum raised to the purified aggregative fimbriae by using immunogold (Fig. 3C). A lack of immunological cross-reactivity among the three fimbrins was also demonstrated by Western blot analysis. The immune serum raised to the aggregative fimbriae did not react to SEF 14 or SEF 21 fimbrins, and conversely, immune sera generated to the SEF 14 and SEF 21 fimbrins did not cross-react with the 17-kDa protein (data not shown).

DISCUSSION

S. enteritidis 27655-3b produces thin, fibrillar surface appendages which morphologically resemble the thin fimbriae found on other bacteria (2, 21, 23, 37, 40–42, 58) but in fact are not specifically related to any other known fimbriae. The thin, aggregative structures produced by *S. enteritidis* 27655-3b are composed mainly of a single protein whose acidic pI and molecular mass of 17 kDa are characteristic of the fimbrin subunits of other bacterial fimbriae (Table 2). Moreover, the total amino acid composition of these thin, aggregative fibrils reveals features common to other fimbriae, including low cysteine and methionine contents and a low percentage of basic and aromatic residues (Table 2). In this regard, the thin fimbriae of *S. enteritidis* 27655-3b are clearly not related to the curli of *E. coli* (Table 2), which themselves appear to be a separate class of fimbriae (42). Furthermore, curli are preferentially expressed on solid CFA medium between 26 and 32°C, whereas the thin aggregative fimbriae of *S. enteritidis* 27655-3b are produced at 30 and 37°C on solid and liquid media. Biochemical data for approximately 50 fimbrial types were analyzed, and none of these fimbriae had the same combination of biochemical characteristics as the thin, aggregative fimbriae of *S. enteritidis* 27655-3b. Relatively few fimbriae have a similar low percentage of hydrophobic residues (Table 2) (2, 8, 14, 31, 59), and only *Haemophilus influenzae* fimbriae (22), type 3 fimbriae of *S. enteritidis* 1380-36/71, and coiled fimbriae of *E. coli* IH11033 share the characteristic of a relatively high percentage of potentially acidic residues (Table 2). Moreover, a FASTA computer search of N-terminal sequence data contained in SWISS-PROT and GENPEPT or our own

TABLE 1. Comparison of N-terminal sequences of fimbrin subunits of aggregative fimbriae isolated from *S. enteritidis* 27655-3b with those of other representative fimbrial types

Organism	Fimbrin	N-terminal amino acid sequence[a]	Reference
S. enteritidis 27655-3b	SEF 17	G V V P Q W G G G G N H N–	
	SEF 14	A G F V G N K A V V Q A A V T I A–	17
	SEF 21 (type 1)	A D P T P V S V S G G T I H F E G–[b]	37a
E. coli	Curlin	T L P S G H L K S R L N K K F T A–[c]	44
		T L P S G H P K S R L I K K F T A–	39
ETEC	CFA/I	V E K N I T V T A S V D P V I D L–	23
ETEC	CFA/II CS3	A A G P T L T K E L A L N V L S P–	23
ETEC	K88	W M T G D F N G S V D I G G S I T–	21
N-MePhe consensus		F*T L I E L M I V x A I x G I L A–[d]	35

[a] See Table 2 for the three-letter code corresponding to the single-letter amino acid assignment.
[b] This sequence is identical to that of type 1 fimbrin from *Salmonella typhimurium* (48).
[c] This sequence was translated from DNA sequences.
[d] F*, N-MePhe; x, either I or V depending on the organism from which the fimbriae were isolated.

TABLE 2. Total amino acid compositions of thin fimbriae from *S. enteritidis* 27655-3b compared with compositions of representative fimbriae[a]

Organism and fimbrial subunit	No. of amino acid residues																	% of residues				Size (kDa)	Diam (nm)	pI	Reference(s)
	D/N (Asp/Asn)	T (Thr)	S (Ser)	E/Q (Glu/Gln)	P (Pro)	G (Gly)	A (Ala)	C (Cys)	V (Val)	M (Met)	I (Ile)	L (Leu)	Y (Tyr)	F (Phe)	H (His)	K (Lys)	R (Arg)	W (Trp)	Acidic	Basic	Hydrophobic[b]	Aromatic[c]	Polar uncharged			
S. enteritidis 27655-3b																										
SEF 17	31	11	13	17	3	26	19	0[d]	10	1	6	7	6	3	1	3	4	+1[e]	30	5	34	6	35	17	3–4	4.5–4.9	
SEF 14[f]	13	16	10	13	7	20	20	0	16	0	6	4	2	7	1	4	3	2	18	6	43	8	33	14	<5	ND	17, 19a
SEF 21 (type 1)	25	25	16	14	12	16	33	ND	16	3	8	13	4	8	1	8	4	ND	19	6	47	6	30	21	6–7	ND	37a
S. enteritidis	30	21	24	23	0	18	22	1	11	1	6	13	5	5	1	6	2	1	28	5	33	6	36	23.5	2	3.9	2
E. coli																											
Curlin	12	8	5	16	7	6	7	3	9	1	1	16	3	10	3	12	9	4	21	18	41	13	19	17	2	NA	42, 44
CS3	23	27	16	6	5	8	13	0	9	0	9	15	1	3	2	6	1	1	20	6	38	3	36	15.3	2	NA	23
K88ab	30	29	18	17	5	36	28	0	21	3	13	20	10	11	1	11	8	4	18	7	42	9	35	27.5	2	4.2	21
F1845	20	21	6	9	2	19	10	2	10	1	4	11	6	2	26	7	3	3	21	9	33	8	39	14.3	2	NA	8
IH11033	28	23	5	20	7	23	11	0	17	0	6	12	0	4	0	5	tr	ND	30	3	35	2	32	16.3	NA	NA	58

[a] ND, not determined; NA, data not available.
[b] Assuming composition of P, A, V, M, I, L, Y, and F.
[c] Assuming composition of G, S, T, C, and Y.
[d] No cysteine detected by analysis of the carboxymethylated thin, aggregative fimbriae.
[e] Tryptophan analysis was not done, but the N-terminal amino acid sequence contains one residue.
[f] Calculated from the translated DNA sequence.

comparison of 40 fimbrial sequences obtained from the literature did not reveal N-terminal sequences homologous to those of the *S. enteritidis* 27655-3b thin fimbriae. The N terminus of the *S. enteritidis* 27655-3b thin, aggregative fimbria resembled those of other fimbriae in that it was composed mainly of polar uncharged or hydrophobic residues. However, it had a unique sequence which was unusual because of the predominance of glycine residues (Table 1). The thin, aggregative fimbriae are biochemically and serologically distinct from SEF 14 and SEF 21, the other two fimbrial types produced by *S. enteritidis* 27655-3b (37a), and represent the third fimbrial type isolated from this enteric pathogen (Tables 1 and 2). Accordingly, these thin fimbriae have been given the trivial name SEF 17 (*S. enteritidis* fimbriae composed of 17-kDa fimbrin subunits).

The detailed biochemical characterization of SEF 17 establishes that these thin fimbriae of *S. enteritidis* 27655-3b are novel with respect to fimbriae from other bacteria. The features which clearly set SEF 17 apart from any other fimbriae are their extreme insolubility and aggregative nature and the difficulty encountered in separating them from the cells. Fimbriae are routinely released from cells by blending, which serves as the first step in most purification schemes (2, 8–10, 14, 17, 22, 24, 25, 29, 30, 37, 58). Occasionally, moderate heat treatment of cells has also been used to release fimbriae (5, 21, 60). Normally, fimbriae released by either method remain predominantly in the supernatant after the cells have been removed by centrifugation. A subsequent salt-, pH-, or solvent-induced precipitation step is required to aggregate the fimbriae and allow their further purification from the supernatant (4, 9, 17, 25, 30, 42). In order to purify the thin, aggregative fimbriae from *S. enteritidis* 27655-3b, an unconventional procedure was used because of the difficulty encountered in separating the fimbriae from the cells. The extreme insolubility of these fimbriae was used to advantage, since virtually all other cell macromolecules could be removed by enzymatic digestion of sonicated cells with a combination of RNase, DNase, and lysozyme followed by solubilization of contaminants with SDS and SDS-PAGE sample buffer and separation of the contaminants from the fimbriae by preparative electrophoresis. Analysis of the aggregative fimbriae by SDS-PAGE of samples conventionally prepared for electrophoresis was not possible, since the fimbriae required a brief treatment with 90% formic acid to promote depolymerization. Normally, boiling in SDS-PAGE sample buffer is sufficient for depolymerization of fimbriae prior to analysis by SDS-PAGE, although the flexible fimbriae of *Aeromonas hydrophila* are somewhat insoluble under these conditions (25). Type 1 fimbriae require mild acidic treatment (H_2O adjusted to pH 1.8; 0.01 M HCl) at 100°C and boiling in SDS-PAGE sample buffer to become depolymerized (36). This acidic treatment was sufficient for depolymerization of type 1 fimbriae (SEF 21) of *S. enteritidis* 27655 (37a), but it failed to depolymerize the thin, aggregative fimbriae of the same bacterium.

Importantly, the results of this study indicate that the use of conventional purification schemes would not result in the detection of similar aggregative fimbriae from other bacteria, since such fimbriae would be discarded with the cell pellet after the initial blending step. Moreover, commonly used electrophoresis methods would ensure that any small fimbrial fragments released by blending of the cells but not removed by centrifugation would be left in the well of the polyacrylamide gel following electrophoresis.

The aggregative nature of the thin fimbriae from *S. enteritidis* 27655-3b is not due to an unusually high hydrophobic

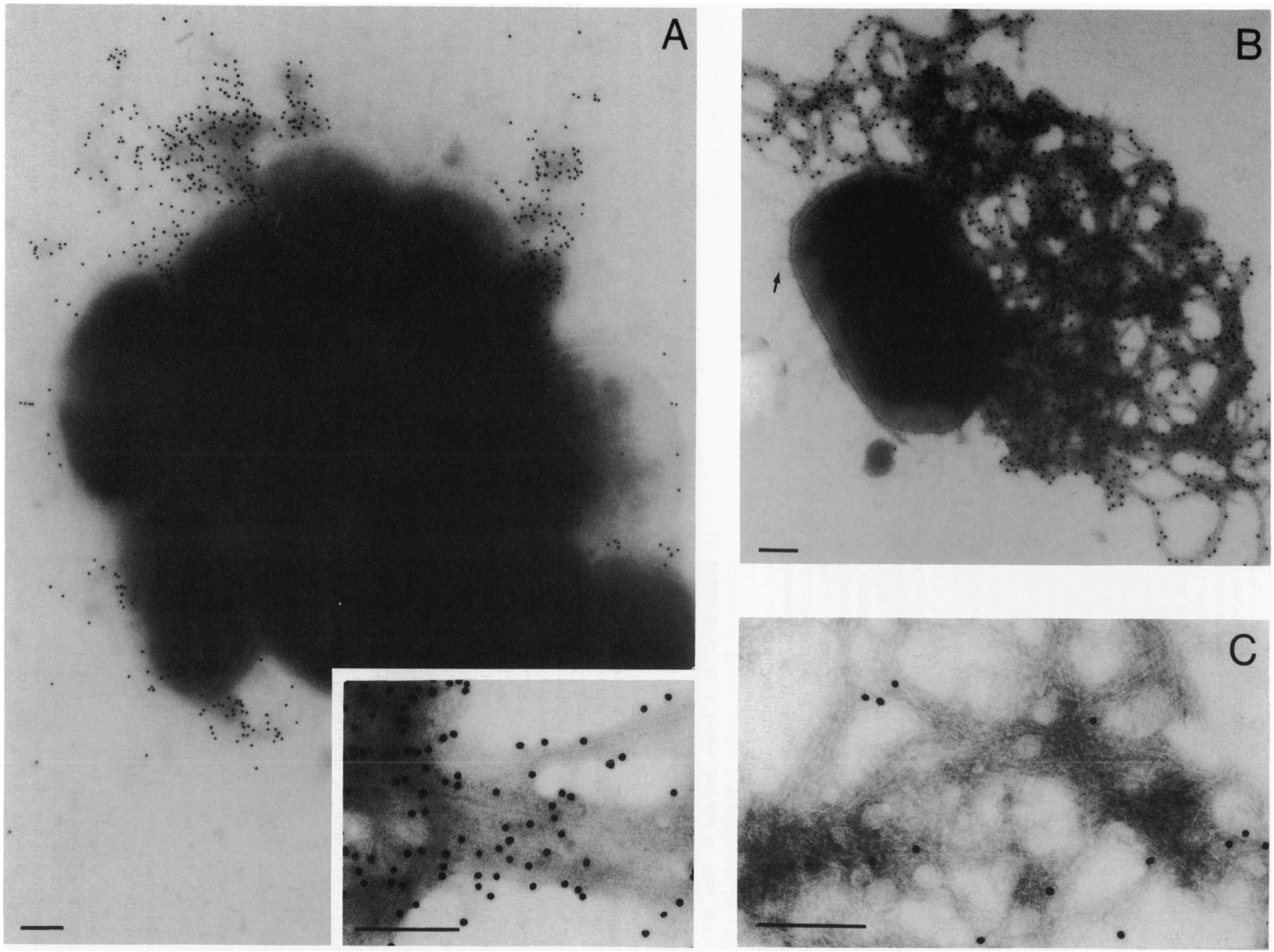

FIG. 3. Electron micrographs of immunogold-labeled native *S. enteritidis* 27655-3b fimbriae. (A) Whole cells isolated from T medium; inset, detail of labeled thin, aggregative fimbriae (SEF 17). (B) Whole cells grown in liquid CFA medium, static for 48 h at 30°C. Arrow indicates type 1 fimbriae (SEF 21). (C) Purified SEF 14 fimbriae. Bars, 200 nm. Whole cells and purified SEF 14 were incubated with immune serum to SEF 17 prior to labeling with protein A-gold.

amino acid composition, since fimbrins from other fimbriae usually have a significantly higher percentage of such residues (Table 2) (21, 23, 25, 37, 45, 48). The secondary and tertiary structures of the fimbrin and its polymerized form likely define the overall hydrophobicity of the fibrils and their chemical resistance. In this regard, it is interesting that the bacterial surface layer of *Methanospirillum hungatei* has a similar resistance to denaturation and depolymerization by alkali, detergents, and chaotropic agents; is solubilized in concentrated performic acid; and has a very similar amino acid composition (7). Sequence determination and further structural analysis of the 17-kDa fimbrin of *S. enteritidis* 27655-3b will undoubtedly reveal some interesting insights into the assembly and structure of these fimbriae.

The function of SEF 17 is unknown, but it is apparent that these fimbriae play a role in cell aggregation and possibly in tissue colonization. The extreme autoaggregative characteristic of the enterotoxigenic *S. enteritidis* strain is abolished in transposon mutants unable to produce these thin fimbriae, and, unlike the wild type, these mutants are unable to bind the tissue matrix proteins fibronectin and laminin (11a). Consequently, a bifunctional role for these fimbriae in virulence can be envisioned. These enteropathogens would have a mechanism for surviving the extremes of pH, presence of digestive enzymes, and host bacteriocidal factors (4, 52) encountered if they travelled through the digestive tract in aggregates. This would ensure that a viable and sufficient inoculum would reach the intestinal epithelium, where the fimbriae could assist in colonization of the host tissue to establish an infection (4). Furthermore, large aggregates of *S. enteritidis* may escape phagocytosis. It is of interest that the molecular basis for enteroadherent-aggregative *E. coli* has been elusive (34, 61). If an aggregative fimbrial type analogous to that found on *S. enteritidis* 27655-3b is present on enteroadherent-aggregative *E. coli*, then purification of such structures may require isolation techniques analogous to those described herein.

ACKNOWLEDGMENTS

We thank S. Kielland, who performed the amino acid analysis and N-terminal sequencing; J. Austin, who did the FASTA search; R. Garduño, who provided useful discussions; and J. Doran, who critically read the manuscript.

The Natural Sciences and Engineering Research Council of Canada (NSERC) provided funding to S.K.C. in the form of a postdoctoral fellowship. L.E. was the recipient of an International Scientific Exchange Award from NSERC. This project was supported by an operating grant to W.W.K. provided by the British Columbia Health Care Research Foundation.

REFERENCES

1. **Adegbola, R. A., D. C. Old, and S. Aleksic.** 1983. Rare MR/K-like haemagglutinins (and type 3-like fimbriae) of *Salmonella* strains. FEMS Microbiol. Lett. **19**:233–238.
2. **Aleksic, S., and V. Aleksic.** 1979. Purification and physicochemical analysis of the fimbrial antigen in two different genera of enterobacteriaceae: *Salmonella enteritidis* and *Yersinia enterocolitica*. Zentralbl. Bakteriol. Hyg. Abt. I Orig. Reihe A **243**:177–196.
3. **Ames, G. F.-L.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249**:634–644.
4. **Arp, L. H.** 1988. Bacterial infection of mucosal surfaces: an overview of cellular and molecular mechanisms, p. 3–27. *In* J. A. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
5. **Baloda, S. B.** 1988. Characterization of fibronectin binding to *Salmonella enteritidis* strain 27655R. FEMS Microbiol. Lett. **49**:483–488.
6. **Baloda, S. B., A. Faris, and K. Krovacek.** 1988. Cell-surface properties of enterotoxigenic and cytotoxic *Salmonella enteritidis* and *Salmonella typhimurium*: studies on hemagglutination, cell-surface hydrophobicity, attachment to human intestinal cells and fibronectin-binding. Microbiol. Immunol. **32**:447–459.
7. **Beveridge, T. J., M. Stewart, R. J. Doyle, and G. D. Sprott.** 1985. Unusual stability of the *Methanospirillum hungatei* sheath. J. Bacteriol. **162**:728–737.
8. **Bilge, S. S., C. R. Clausen, W. Lau, and S. L. Moseley.** 1989. Molecular characterization of a fimbrial adhesin, F1845, mediating diffuse adherence of diarrhea-associated *Escherichia coli* to HEp-2 cells. J. Bacteriol. **171**:4281–4289.
9. **Brinton, C. C., Jr.** 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Trans. N.Y. Acad. Sci. **27**:1003–1054.
10. **Broes, A., J. M. Fairbrother, N. Jacques, and S. Larivière.** 1988. Isolation and characterization of a new fimbrial antigen (CS1541) from a porcine enterotoxigenic *Escherichia coli* O8:KX105 strain. FEMS Microbiol. Lett. **55**:341–348.
11. **Clegg, S., and G. F. Gerlach.** 1987. Enterobacterial fimbriae. J. Bacteriol. **169**:934–938.

11a. **Collinson, S. K., L. Emödy, K.-H. Müller, P. C. Doig, W. W. Kay, and T. J. Trust.** Unpublished data.

12. **Cooke, E. M.** 1990. Epidemiology of foodborne illness: UK. Lancet **336**:790–793.
13. **Crichton, P. B., D. E. Yakubu, D. C. Old, and S. Clegg.** 1989. Immunological and genetical relatedness of type-1 and type-2 fimbriae in salmonellas of serotypes gallinarum, pullorum and typhimurium. J. Appl. Bacteriol. **67**:283–291.
14. **de Graaf, F. K., and I. Roorda.** 1982. Production, purification, and characterization of the fimbrial adhesive antigen F41 isolated from calf enteropathogenic *Escherichia coli* strain B41M. Infect. Immun. **36**:751–758.
15. **Engval, E., and P. Perlmann.** 1972. Enzyme-linked immunosorbent assay, ELISA. J. Immunol. **109**:129–135.
16. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
17. **Feutrier, J., W. W. Kay, and T. J. Trust.** 1986. Purification and characterization of fimbriae from *Salmonella enteritidis*. J. Bacteriol. **168**:221–227.
18. **Feutrier, J., W. W. Kay, and T. J. Trust.** 1988. Cloning and expression of *Salmonella enteritidis* fimbrin genes in *Escherichia coli*. J. Bacteriol. **170**:4216–4222.
19. **Feutrier, J., W. W. Kay, T. Wadström, and T. J. Trust.** 1986. Novel type 1 fimbriae of *Salmonella enteritidis*, p. 103–109. *In* D. L. Lark (ed.), Protein-carbohydrate interactions in biological systems. Academic Press, Inc. (London), Ltd., London.

19a. **Feutrier, J., T. J. Trust, and W. W. Kay.** Unpublished data.

20. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53**:210–230.
21. **Gaastra, W., and F. K. de Graaf.** 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. Microbiol. Rev. **46**:129–161.
22. **Guerina, N. G., S. Langermann, G. K. Schoolnik, T. W. Kessler, and D. A. Goldmann.** 1985. Purification and characterization of *Haemophilus influenzae* pili, and their structural and serological relatedness to *Escherichia coli* P and mannose-sensitive pili. J. Exp. Med. **161**:145–159.
23. **Hall, R. H., D. R. Maneval, Jr., J. H. Collins, J. L. Theibert, and M. M. Levine.** 1989. Purification and analysis of colonization factor antigen I, coli surface antigen 1, and coli surface antigen 3 fimbriae from enterotoxigenic *Escherichia coli*. J. Bacteriol. **171**:6372–6374.
24. **Heuzenroeder, M. W., T. R. Elliot, C. J. Thomas, R. Halter, and P. A. Manning.** 1990. A new fimbrial type (PCFO9) on enterotoxigenic Escherichia coli 09:H^-LT^+ isolated from a case of

infant diarrhea in central Australia. FEMS Microbiol. Lett. 66:55–60.
25. **Ho, A. S. Y., T. A. Mietzner, A. J. Smith, and G. K. Schoolnik.** 1990. The pili of *Aeromonas hydrophila*: identification of an environmentally regulated "mini-pilin." J. Exp. Med. **172**:795–806.
26. **Humphrey, T. J., A. Baskerville, S. Mawer, B. Rowe, and S. Hopper.** 1989. *Salmonella enteritidis* phage type 4 from the contents of intact eggs: a study involving naturally infected hens. Epidemiol. Infect. **103**:415–423.
27. **Isaacson, R. E.** 1988. Molecular and genetic basis of adherence for enteric *Escherichia coli* in animals, p. 28–44. *In* J. A. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
28. **Jones, B. N., J. E. Shively, D. L. Kilpatrick, A. S. Stern, R. V. Lewis, K. Kojima, and S. Udenfriend.** 1982. Adrenal opioid proteins of 8,600 and 12,600 daltons: intermediates in proenkephalin processing. Proc. Natl. Acad. Sci. USA **171**:2096–2100.
29. **Korhonen, T. K., K. Lounatama, H. Ranta, and N. Kuusi.** 1980. Characterization of type 1 pili of *Salmonella typhimurium* LT2. J. Bacteriol. **144**:800–805.
30. **Korhonen, T. K., E.-L. Nurmiaho, H. Ranta, and C. S. Edén.** 1980. New method for isolation of immunologically pure pili from *Escherichia coli*. Infect. Immun. **27**:569–575.
31. **Korhonen, T. K., E. Tarkka, H. Ranta, and K. Haahtela.** 1983. Type 3 fimbriae of *Klebsiella* sp.: molecular characterization and role in bacterial adhesion to plant roots. J. Bacteriol. **155**:860–865.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
33. **LeGendre, N., and P. Matsudaira.** 1988. Direct protein microsequencing from Immobilon™-P transfer membrane. Biotechniques **6**:154–159.
34. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155**:377–389.
35. **Mattick, J. S., M. M. Bills, B. J. Anderson, B. Dalrymple, M. R. Mott, and J. R. Egerton.** 1987. Morphogenetic expression of *Bacteroides nodosus* fimbriae in *Pseudomonas aeruginosa*. J. Bacteriol. **169**:33–41.
36. **McMicheal, J. C., and J. R. Ou.** 1979. Structure of common pili from *Escherichia coli*. J. Bacteriol. **138**:969–975.
37. **Moriya, T., S.-I. Kawabata, Y. Mizunoe, and K. Amako.** 1989. A cryptic fimbrial gene in *Serratia marcescens*. J. Bacteriol. **171**:6629–6636.
37a. **Müller, K.-H., S. K. Collinson, T. J. Trust, and W. W. Kay.** 1991. Type 1 fimbriae of *Salmonella enteritidis*. J. Bacteriol. **173**:4765–4772.
38. **Müller, K.-H., T. J. Trust, and W. W. Kay.** 1989. Fimbriation genes of *Salmonella enteritidis*. J. Bacteriol. **171**:4648–4654.
39. **Nüesch, J., and D. Schümperli.** 1984. Structural and functional organization of the *gpt* gene region of *Escherichia coli*. Gene **32**:243–249.
40. **Old, D. C., A. Tavendale, and B. W. Senior.** 1983. A comparative study of the type-3 fimbriae of *Klebsiella* species. J. Med. Microbiol. **20**:203–214.
41. **Old, D. C., A. Tavendale, and D. E. Yakubu.** 1989. Some strains of *Escherichia coli* of putative enteroadherent-aggregative serotypes produce an unusual fibrillar haemagglutinin. FEMS Microbiol. Lett. **59**:87–92.
42. **Olsen, A., A. Jonsson, and S. Normark.** 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature (London) **338**:652–655.
43. **Ottow, J. C. G.** 1975. Ecology, physiology, and genetics of fimbriae and pili. Annu. Rev. Microbiol. **29**:79–108.
44. **Overbeeke, N., H. Bergmann, F. van Mansfeld, and B. Lugtenberg.** 1983. Complete nucleotide sequence of *phoE*, the structural gene for the phosphate limitation inducible outer membrane pore protein of *Escherichia coli* K12. J. Mol. Biol. **163**:513–532.
45. **Paranchych, W.** 1990. Molecular studies on *N*-methylphenylalanine pili, p. 61–78. *In* B. H. Iglewski and V. L. Clark. (ed.), Molecular basis of bacterial pathogenesis. Academic Press, Inc., New York.
46. **Paranchych, W., and L. S. Frost.** 1988. The physiology and biochemistry of pili. Adv. Microb. Physiol. **29**:53–114.
47. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
48. **Purcell, B. K., J. Pruckler, and S. Clegg.** 1987. Nucleotide sequences of the genes encoding type 1 fimbrial subunits of *Klebsiella pneumoniae* and *Salmonella typhimurium*. J. Bacteriol. **169**:5831–5834.
49. **Robertson, E. F., H. K. Dannelly, P. J. Malloy, and H. C. Reeves.** 1987. Rapid isoelectric focusing in a vertical polyacrylamide minigel system. Anal. Biochem. **167**:290–294.
50. **Rodrigue, D. C., R. V. Tauxe, and B. Rowe.** 1990. International increase in *Salmonella enteritidis*: a new pandemic? Epidemiol. Infect. **105**:21–27.
51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1982. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Savage, D. C.** 1987. Factors influencing biocontrol of bacterial pathogens in the intestine. Food Technol. **41**:82–87.
53. **Stocker, B. A. D., and P. H. Mäkelä.** 1986. Genetic determination of bacterial virulence, with special reference to *Salmonella*. Curr. Top. Microbiol. Immunol. **124**:149–172.
54. **Swank, R. T., and K. D. Munkres.** 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal. Biochem. **39**:462–477.
55. **Tacket, C. O., D. R. Maneval, and M. M. Levine.** 1987. Purification, morphology, and genetics of a new fimbrial putative colonization factor of enterotoxigenic *Escherichia coli* O159:H4. Infect. Immun. **55**:1063–1069.
56. **Tennent, J. M., S. Hultgren, B.-I. Marklund, K. Forsman, M. Göransson, B. E. Uhlin, and S. Normark.** 1990. Genetics of adhesin expression in *Escherichia coli*, p. 79–110. *In* B. H. Iglewski and V. L. Clark. (ed.), Molecular basis of bacterial pathogenesis. Academic Press, Inc., New York.
57. **Todd, E.** 1990. Epidemiology of foodborne illness: North America. Lancet **336**:788–790.
58. **Väisänen-Rhen, V.** 1984. Fimbria-like hemagglutinin of *Escherichia coli* O75 strains. Infect. Immun. **46**:401–407.
59. **van Ham, S. M., F. R. Mooi, M. G. Sindhunata, W. R. Maris, and L. van Alphen.** 1989. Cloning and expression in *Escherichia coli* of *Haemophilus influenzae* fimbriae genes establishes adherence to oropharyngeal epithelial cells. EMBO J. **8**:3535–3540.
60. **van Zijderveld, F. G., J. Anakotta, R. A. M. Brouwers, A. M. van Zijderveld, D. Bakker, and F. K. de Graaf.** 1990. Epitope analysis of the F4 (K88) fimbrial antigen complex of enterotoxigenic *Escherichia coli* by using monoclonal antibodies. Infect. Immun. **58**:1870–1878.
61. **Vial, P. A., R. Robins-Browne, H. Lior, V. Prado, J. B. Kaper, J. P. Nataro, K. Maneval, A. Elsayed, and M. M. Levine.** 1988. Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. J. Infect. Dis. **158**:70–79.
62. **Yakubu, D. E., B. W. Senior, and D. C. Old.** 1989. A novel fimbrial haemagglutinin produced by a strain of *Salmonella* of serotype Salinatis. FEMS Microbiol. Lett. **57**:29–34.